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Post-harvest and scale-up extraction of American mayapple leaves for podophyllotoxin production

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Abstract

Leaves of American mayapple (*Podophyllum peltatum* L.*)* are of interest to the pharmaceutical industry as an alternative source of podophyllotoxin, an aryltetralin lignan that is the precursor of widely used anticancer drugs etoposide, teniposide, and etopophos. In this study, the effects of post-harvest handling were evaluated by inflicting physical damage to leaves of mayapple to simulate rough handling. The effects of storage conditions before and after being dried were also evaluated. In addition, techniques for conducting large-scale extractions of podophyllotoxin from bulk samples were investigated. Crushing injury, damaging the leaves in more than 70% of its area, has improved podophyllotoxin content of leaves when dried at 40 °C within 24 h of harvest. In contrast, podophyllotoxin content was greatly reduced when the leaves were dried at room temperature at 15% relative humidity and 24 °C. Podophyllotoxin was stable with no significant changes over time when the leaves were dried, ground, and stored under different conditions for up to 60 days. Based on these findings, mayapple leaves do not require careful handling at harvest. In fact, leaves can be handled in a manner consistent with mechanical injury as long as leaves are dried at 40 °C within 24 h. Leaves can then be stored for up to 60 days, and probably much longer, when dry. If leaves cannot be dried in a timely manner, they can be stored at 4 °C for up to 4 weeks without significant loss of podophyllotoxin.

Keywords: Podophyllum; Lignans; Biomass; Handling; Drying

1. Introduction

Podophyllotoxin is a natural lignan found in *Podophyllum* species (Berberidaceae), and is the starting compound for the semi-synthesis of anticancer pharmaceuticals such as etoposide, etopophos, and teniposide (Stahelin and von Wartburg, 1991). These neoplastic compounds block DNA topoisomerase II (Loike and

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Horwitz, 1976; Horwitz and Loike, 1977; Minocha and Long, 1984) and have been used for the treatment of small and large cell lung cancer, refractory testicular cancer, stomach and pancreatic cancers, and myeloid leukemias (Ekstrom et al., 1998; Holm et al., 1998; Ajani et al., 1999). Podophyllotoxin is also the precursor to a new derivative, CPH 82 that is being tested for rheumatoid arthritis in Europe (Calstrom et al., 2000). Several dermatological preparations containing podophyllotoxin are on the market for use in the treatment of genital warts (Claesson et al., 1996).

Currently, podophyllotoxin is extracted for commercial purposes from the rhizomes and roots of *Podophyllum emodi* Wall, an endangered species from the Himalayas. Total synthesis of podophyllotoxin was reported to be uneconomical, but leaves of American mayapple (*Podophyllum peltatum* L.) were reported to be an alternative source of the compound (Jackson and Dewick, 1984; Canel et al., 2001). *P. peltatum* grows in large colonies in eastern North America from Quebec and Minnesota to Florida and Texas (Meijer, 1974). A partial survey of natural populations of mayapple resulted in the identification and isolation of several high-yielding accessions (Moraes et al., 2000). Our research efforts for the past 6 years at the National Center for Natural Products Research (NCNPR) have focused on domestication of these high-yielding accessions. We have explored mayapple as an alternative crop for podophyllotoxin and for its potential interest to growers of specialty crops (Moraes et al., 2002; Cushman et al., 2005).

Frequently, plant biomass looses quality during harvest and storage due to the poor handling and physical damages to tissues/cells. Damage induces enzymatic and/or chemical reactions that increase the levels of respiration and transpiration. In addition to poor handling, the logistics of drying large amounts of biomass in a relatively short period of time is difficult. Additionally, conditions are often poor during transport and storage. Drying plants under less than ideal conditions can alter the levels of biologically active components, and thus alter the quality of the commodity. Changes in essential oils are the primary concern in post-harvest physiology of herbs and medicinal plants (Whish and Williams, 1996; Boettcher et al., 1999). Few studies have dealt with other classes of compounds, Wills and Stuart (2000) reported that rough handling of *Echinacea purpurea* had increased the levels of alkamides. The purpose of this study is to evaluate the effects of post-harvest handling and storage conditions related to lignan content of mayapple leaves as well as to examine the scale-up of the extraction procedure begun by our group (Canel et al., 2001).

2. Material and methods

2.1. Plant material

Leaves of *P. peltatum* were harvested from the wild near the University of Mississippi campus in Oxford, MS (N 34°16.387′, W 88°44.689′). A voucher specimen was submitted to the Pullen Herbarium, University of Mississippi, as a reference material.

2.2. Post-harvest handling

Leaves were collected from two adjacent colonies located less than 3 m apart. Twenty leaves per colony were divided into four groups with each group subjected to a unique handling/storage treatment combination. Each group had five leaves per treatment. Treatments were (1) undamaged leaves as the control, (2) leaves chopped into 10 pieces with a sharp knife, (3) leaves crushed by a roller applying 60 kg pressure at room temperature, and (4) leaves crushed by a roller applying 60 kg pressure and held 24 h at room temperature. After performing each treatment, the leaf material was immediately placed in a forced-air dryer at 40 °C for 48 h. Dried leaves were then ground to fine powder using a coffee grinder (Braun®) and immediately analyzed for podophyllotoxin (Canel et al., 2001).

2.3. Drying methods

Ninety undamaged leaves collected from adjacent colonies were divided into nine groups each consisting of five replicates and two leaves per replicate. Drying and storage treatment combinations consisted of (1) dried immediately after harvest in forced-air dryer at 40 °C for 48 h, (2) freeze-dried, (3) dried at room temperature for 6 days, (4) dried in forced-air dryer at 40 °C for 48 h after 24 h storage at room temperature, (5) dried in forced-air dryer at 40 °C for 48 h after 24 h storage at 4 °C, (6) dried in forced-air dryer at 40 °C for 48 h after 1 week storage at 4 °C, (7) dried in forced-air dryer at 40 °C for 48 h after 2 weeks storage at 4 °C, (8) dried in forced-air dryer at 40 °C for 48 h after 3 weeks storage at 4 °C, and (9) dried in forced-air dryer at 40 °C for 48 h after 4 weeks storage at 4 °C.

Dried leaves of all described treatments were ground to fine powder using a coffee grinder (Braun®), and then analyzed for podophyllotoxin.

2.4. Storage of dried leaves

For testing storage conditions of ground biomass, 240 freshly harvested leaves were dried at 40 °C for 48 h and ground to a fine powder. Ground leaves were stored in either clear or amber bottles. Four of each type of bottle was placed in storage at either 5 or 24 °C. After 0, 10, 20, 30, and 60 days of storage, samples (500 mg) were removed in triplicates and analyzed for podophyllotoxin content. Samples taken after each storage time were analyzed and converted into percentage of podophyllotoxin at storage time 0 day.

2.5. Extraction and podophyllotoxin analyses

The aryltetralin lignans (podophyllotoxin, α-peltatin, and β-peltatin) were separated and quantified by HPLC (Canel et al., 2001). Statistical analysis of the data was performed on the original data by one-way analysis of variance (ANOVA) or regression analysis using SPSS 11.5 for Windows.

2.6. Large-scale extraction

Mayapple leaves (50 kg dry matter) were harvested from different colonies located in Lafayette county, Mississippi. They were dried for 24 h at 45 ± 3 °C in a tobacco leaf dryer and grounded to a fine powder using the Retsch grinder Type SM 100. Finely ground dry *P. peltatum* (5.0 kg) was suspended in 80 l of distilled water in a 114 l plastic container. The suspension was stirred with a mechanical stirrer for 30 min at 1700 rpm to ensure complete suspension of the plant material and to allow for incubation. The suspension was transferred in 10 l batches into a large (20 l) round bottom glass flask and washed with hexane (1.8 l × 3) by rotating the flask at 30 rpm for 3 min using a large-scale rotary evaporator with the vacuum off. The hexane layer was removed by aspiration and the aqueous suspension extracted with ethyl acetate (1.8 l × 3), with mixing by rotating the flask at 30 rpm for 3 min. The free ethyl acetate extract supernatants were removed by aspiration through Teflon lines. The aqueous suspension containing a moderate amount of ethyl acetate emulsion was suction-filtered through four layers of cheesecloth set in a Buchner funnel. The filtrate was placed in a separatory funnel and the ethyl acetate layer separated from the aqueous layer. The solids filtered with the cheesecloth were re-extracted with ethyl acetate (20 l) and filtered through filter paper (Whatman #1). All ethyl acetate layers were combined and suction-filtered one final time through filter paper (Whatman #1). The ethyl acetate was removed under reduced pressure at room temperature with a rotary evaporator to yield 230 g of the desired resin consisting of 20.0% podophyllotoxin.

3. Results and discussion

3.1. Post-harvest handling

Podophyllotoxin content was significantly greater in leaves that were crushed with a roller than in leaves that were undamaged or cut with a knife (Table 1). Crushed leaves contained about 16 and 21% more podophyllotoxin than undamaged or cut leaves, respectively. In contrast, crushed leaves that were stored at room temperature for 24 h before drying contained 20% less podophyllotoxin than the leaves that were crushed and dried immediately. Crushed leaves stored at room temperature for 24 h produced equal amounts of podophyllotoxin to that of undamaged and cut leaves (Table 1).

These results indicate that crushing injuries may elicit the production of podophyllotoxin in mayapple leaves. Canel et al. (2001) demonstrated that podophyllotoxin was stored in leaves as glucosides and that rehydratation of dried tissues leads to the conversion of lignan aglycone by in situ β-glucosidase. Crushing fresh leaves could partially rupture the tissues/cells compartmentalization releasing hydrolytic enzymes into the cytoplasm, mimicking a pathogen attack leading to extra production of lignans as a defense response. Chopping, on the other hand, did not affect podophyllotoxin content. Perhaps damage caused by chopping disrupted only a small percentage of total cells throughout the leaf matrix and not enough to enhance lignan production.

Leaves stored at 4 °C for 24 h or for 1, 2, 3, or 4 weeks in airtight bags did not have any significant loss of podophyllotoxin content (Table 2). From these results, it can be recommended that if leaves cannot be dried within

Table 1
Effect of post-harvest handling treatments and storage conditions on podophyllotoxin content in mature leaves of mayapple (*P. peltatum*)

Handling method	Storage conditions	Podophyllotoxin (mg/g)
Undamaged	None	24.2 ± 2.0b
Cut into 10 pieces[a]	None	22.1 ± 3.8b
Crushed with a roller[a]	None	28.1 ± 1.2a
Crushed with a roller[a]	24 h at 24 °C	22.6 ± 1.1b
Significance		0.003

Values followed by the same letter are not significantly different at $P \leq 0.05$. Values are means ± S.D. from five replicates.

[a] Leaves were either cut with a sharp knife or crushed with a roller applying 60 kg of pressure.

Table 2
Effect of storage conditions and drying treatments on podophyllotoxin content in mature leaves of mayapple (*P. peltatum*)

Storage conditions (before drying)	Drying treatments	Podophyllotoxin (mg/g)
None	Forced-air dryer at 40 °C	24.2 ± 2.0a
None	Freeze-dry	19.5 ± 4.3a
None	Room temperature	10.3 ± 2.8b
24 h at room temperature	Forced-air dryer at 40 °C	24.0 ± 4.6a
24 h at 4 °C	Forced-air dryer at 40 °C	24.8 ± 2.4a
1 week at 4 °C	Forced-air dryer at 40 °C	22.0 ± 4.1a
2 weeks at 4 °C	Forced-air dryer at 40 °C	21.3 ± 4.6a
3 weeks at 4 °C	Forced-air dryer at 40 °C	23.2 ± 4.5a
4 weeks at 4 °C	Forced-air dryer at 40 °C	21.6 ± 1.6a
Significance		0.001

Values followed by the same letter are not significantly different at $P \leq 0.05$. Values are means ± S.D. from five replicates.

24 h of harvest, undamaged leaves can be stored at 4 °C for up to 4 weeks without the loss of podophyllotoxin.

3.2. Drying methods

Leaves dried at room temperature over a period of 6 days contained significantly less podophyllotoxin than any of the other handling/drying treatment combinations (Table 2). In addition, drying leaves at room temperature was the only treatment that changed leaf color from green to brown, indicating there was degradation of chlorophyll over time. These results indicate that drying at room temperature is too slow and allows for detrimental changes to occur in leaf tissue before becoming fully desiccated. For maintaining an intense green color of the leaves, freeze-drying showed the best results. Forced-air dried leaves changed color only slightly, becoming slightly pale green in color.

3.3. Storage of dried leaves

Podophyllotoxin content of dried mayapple leaves was stable throughout a 60-day period under all but one of the storage conditions. When leaves were stored at 5 °C under light, a 12% loss of the original podophyllotoxin content occurred over the 60-day storage period. The rate of loss was described by a quadratic regression, $y = 0.0095x^2 - 722.92x + 1E+07$. It is a common practice in our laboratory to store dried mayapple leaves in sealed dark bottles, and these samples have been stored for more than 3 years with little change in the content of podophyllotoxin and related lignans over time (data not shown). The amber bottles reduce light by 73.9% avoiding changes in the biomass. This experiment is the first evidence in support of our typical laboratory procedures.

3.4. Large-scale extraction

Scale-up of the original extraction conditions as described by Canel et al. (2001) was not feasible with size greater than a few grams. Problems were encountered once the scale reached a point that centrifugation of solids was not practical. The presence of these solids led to the formation of emulsions preventing the separation of the ethyl acetate from the aqueous phase and from the solids. Attempted filtration of these mixtures was not feasible because filtering media (paper, sand, celite, cheesecloth) quickly became clogged or allowed the whole mixture to pass through.

We established that podophyllotoxin was soluble in mixtures of up to 20% water in methanol and that such mixtures (solvent/extract/plant material) could be filtered through paper when extracting at 100 g scales, but at extremely slow rates. Unfortunately, the minimum ratio of methanol needed varied with the amount of extraneous material present so that the rate of filtration slowed considerably at the higher scales of plant extraction, and the amount of methanol calculated as necessary for the extraction of multi-kilogram amounts would have been excessive (e.g., 100 l aqueous extract would have necessitated a minimum of 500 l of methanol).

We also found that podophyllotoxin is soluble in boiling water and that the mixture (solvent/extract/plant material) can be filtered. Unfortunately, podophyllotoxin decomposes rapidly under these conditions and this practice became impractical at the higher scales (≥100 g). Lowering the temperature to 60 °C, podophyllotoxin is still soluble, but slowers the filtration rate. This led to unacceptable decomposition due to the increased amount of time that podophyllotoxin is exposed to such temperatures.

Finally, we were able to establish that washing the aqueous extract with a relatively small amount of hexane prevented the formation of excessive amounts of emulsion on ethyl acetate extraction and allowed most of the ethyl acetate to be removed from the aqueous phase. The remaining water and emulsion could be filtered readily through four layers of cheesecloth to remove most of the solids, allowing the separation of the remaining ethyl acetate from the water phase. These ethyl acetate phases, which contained finely dispersed solids, could then be readily filtered through paper to yield a solid free ethyl acetate extract. The solids filtered with cheesecloth could then be re-extracted with ethyl acetate and re-filtered through paper. Combining all the ethyl acetate

phases then produces a resin that contains podophyllotoxin in a yield that compares favorably (0.93% versus 0.66% podophyllotoxin from plant dry weight) with the original small-scale extractions.

4. Conclusion

This study can be summarized as follows: (1) podophyllotoxin extraction increased with crushing injury or remained the same with cutting injury in freshly harvested mayapple leaves subjected to mechanical damage during handling. These results indicate that leaves can be harvested from plants without concern with rough handling that will lower podophyllotoxin content. (2) Drying the leaves in a forced-air dryer at 40 °C for 48 h and freeze-drying are the two best methods for preserving the podophyllotoxin of mayapple leave tissue. Avoid slow drying at room temperature. (3) When drying facilities are not available within 24 h after harvest, undamaged leaves can be stored at 4 °C for several weeks with minimal loss of podophyllotoxin. (4) Dried leaf tissue stored in airtight glass containers can be stored at 24 °C in the dark for up to 60-day and probably longer, without loss of podophyllotoxin content. (5) The procedure described by Canel et al. (2001) can be applied to large-scale extraction of podophyllotoxin from bulk quantities of mayapple leaves by washing with small amounts of hexane to prevent formation of emulsions.

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